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A role for *Drosophila* SMC4 in the resolution of sister chromatids in mitosis

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Background: Faithful segregation of the genome during mitosis requires interphase chromatin to be condensed into well-defined chromosomes. Chromosome condensation involves a multiprotein complex known as condensin that associates with chromatin early in prophase. Until now, genetic analysis of SMC subunits of the condensin complex in higher eukaryotic cells has not been performed, and consequently the detailed contribution of different subunits to the formation of mitotic chromosome morphology is poorly understood.

Results: We show that the SMC4 subunit of condensin is encoded by the essential *gluon* locus in *Drosophila*. DmSMC4 contains all the conserved domains present in other members of the structural-maintenance-of-chromosomes protein family. DmSMC4 is both nuclear and cytoplasmic during interphase, concentrates on chromatin during prophase, and localizes to the axial chromosome core at metaphase and anaphase. During decondensation in telophase, most of the DmSMC4 leaves the chromosomes. An examination of *gluon* mutations indicates that SMC4 is required for chromosome condensation and segregation during different developmental stages. A detailed analysis of mitotic chromosome structure in mutant cells indicates that although the longitudinal axis can be shortened normally, sister chromatid resolution is strikingly disrupted. This phenotype then leads to severe chromosome segregation defects, chromosome breakage, and apoptosis.

Conclusions: Our results demonstrate that SMC4 is critically important for the resolution of sister chromatids during mitosis prior to anaphase onset.

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Background

Chromosome condensation serves not only to package the long DNA molecules present in eukaryotic cells but also to organize the sister strands into chromatids that can be resolved from one another and from other chromosomes [1, 2]. Early work indicated that DNA topoisomerase II (topo II) was a structural component of mitotic chromosomes [3, 4]. While topo II activity was also shown to be essential for establishing mitotic chromosome condensation both in vivo [5] and in vitro [6, 7], it may not be required to maintain the condensed state [8].

New insights into chromosome dynamics came with the identification of a novel family of conserved chromosomal ATPases, the SMC proteins, reviewed in [9, 10]. SMCs were initially identified in *S. cerevisiae* [11] and in the analysis of the ScII protein that, like topo II, is a component of the chromosome scaffold fraction [12]. SMCs have been identified in all eukaryotic organisms examined to date and fall into six clearly discernible subfamilies

(SMC1–4, Rad18, and Rad18 related). All SMC proteins are large polypeptides that share partial sequence conservation and similar structural organization [9]. The N-terminal end contains a putative Walker A motif (ATP binding domain), and at the C-terminal end is located a characteristic “DA” box (Walker B motif) involved in ATP hydrolysis [12]. These domains are separated by a coiled-coil region, interrupted by a central globular hinge region. A complex containing a heterodimer of SMC2 and SMC4 has been shown to function in chromosome condensation [13–16], while an analogous complex containing an SMC1/SMC3 heterodimer plays a role in sister chromatid cohesion [17–19].

The “condensin” complex, first identified in *Xenopus* egg extracts, is essential for mitosis-specific chromatin condensation in vitro [13]. XCAP-C and XCAP-E, the *Xenopus* SMC2/SMC4 homologs, along with three non-SMC proteins (XCAP-D2, XCAP-G, and XCAP-H) comprise a 13S condensin complex, while an 8S complex consists of

XCAP-C and XCAP-E alone (not sufficient for mitotic chromosome condensation) [20]. A similar five-subunit complex has been identified in fission yeast, and mutations in the non-SMC subunits produce hypocondensed chromosomes that extend along an elongated spindle [16]. The *Xenopus* 13S condensin complex induces ATP-dependent positive supercoiling of a DNA template as a result of its stoichiometric binding to DNA [21, 22]. The targeting of the complex and its supercoiling activity depend on CDK-dependent, mitosis-specific phosphorylation of XCAP-D2 and XCAP-H [23]. The ATP-dependent positive DNA supercoiling that is associated with the condensin complex is proposed to be the driving force for chromatin condensation [21]. In the presence of topo II, condensin converts circular plasmid DNA preferentially into positive trefoil knots, and this observation implies that the condensin complex might generate an ordered array of positive solenoidal supercoils [22]. However, the mechanism by which the *in vitro* DNA supercoiling activity of “condensin” may contribute to the overall condensation mechanism of protein-laden chromosomes is unknown.

The advantages of combined genetic and cytological analyses make *Drosophila* an ideal system for studying the mechanics and regulation of mitosis during development. Of the condensin subunits, so far only the *Drosophila* homolog of XCAP-H (Barren) has been reported [24]. *barren* mutations exhibit defects in the segregation of chromosomes in embryonic neuronal precursor cells. The centromeres separate, but chromosome arms do not resolve, and they show chromatin bridges during anaphase. Examination of the cellular phenotype of *barren* mutants revealed a morphology similar to that exhibited by *top2* (topo II) mutants of *S. cerevisiae* [25] or *cut3* or *cut14* (SMC4 and 2, respectively) mutants of *S. pombe* [14]. The observation that Barren and topo II coimmunoprecipitate and interact in a two-hybrid assay leads to the hypothesis that Barren might modulate topo II activity [24]. However, in budding yeast, the Barren homolog (BRN1) does not appear to be an essential activator of DNA topo II but is an important factor in the establishment and maintenance of chromosome condensation [26, 27].

Here we report the molecular cloning, distribution, and functional analysis of the *Drosophila* ortholog of the SMC4 subunit of condensin. We have characterized two P element insertion alleles, corresponding to the previously described *gluon* locus [28], which cause lethality at different times in development. Mutations in *gluon* cause abnormal chromosome condensation, leading to chromosome breakage and missegregation during mitosis in both embryonic neuronal cells and larval neuroblasts. Detailed analysis of chromosome morphology in mutant cells suggests that SMC4 plays a crucial role in the final stages of mitotic condensation. Strikingly, in *DmSMC4* mutants,

the longitudinal axis of a chromosome can be shortened, while width-wise chromatid compaction and sister chromatid resolution are severely disrupted.

Results

Molecular cloning and characterization of *DmSMC4*

We have cloned a cDNA molecule containing an open reading frame (ORF) encoding a protein of 1409 amino acids with a predicted molecular weight of 160 kDa. The predicted structure of the protein closely resembles that described for other SMC proteins. Phylogenetic analysis of the protein sequence showed that it belonged to the SMC4-type protein family (see the Supplementary material available with this article on the internet). The *Drosophila* protein is closely related to other SMC4-type proteins, including *Xenopus* XCAP-C (41% identity), human HCAP-C (39% identity), *S. cerevisiae* SMC4 (35% identity), and *S. pombe* CUT3 (34% identity) orthologs, and it is more distantly related to other SMC-type proteins. Accordingly, we have named the gene *DmSMC4*.

Genomic organization and molecular characterization of mutant alleles

An examination of the corresponding genomic sequence (AC005119) allowed us to determine the genomic organization of the locus (Figure 1a). Database searches identified one embryonic-lethal P element insertion allele, *l(2)k08819*, that had been previously named *gluon* [28]. Subsequently, we identified a late larval-lethal P element insertion allele, *l(2)k06821*. We have named the alleles *glu¹* and *glu²*, respectively. The localization of the P elements in *glu¹* and *glu²* are indicated in Figure 1a. Molecular characterization of the *glu¹* excision allele *glu⁸⁸⁻⁸²* was carried out by the sequence analysis of PCR products. In *glu⁸⁸⁻⁸²* a large internal region of the P element is deleted.

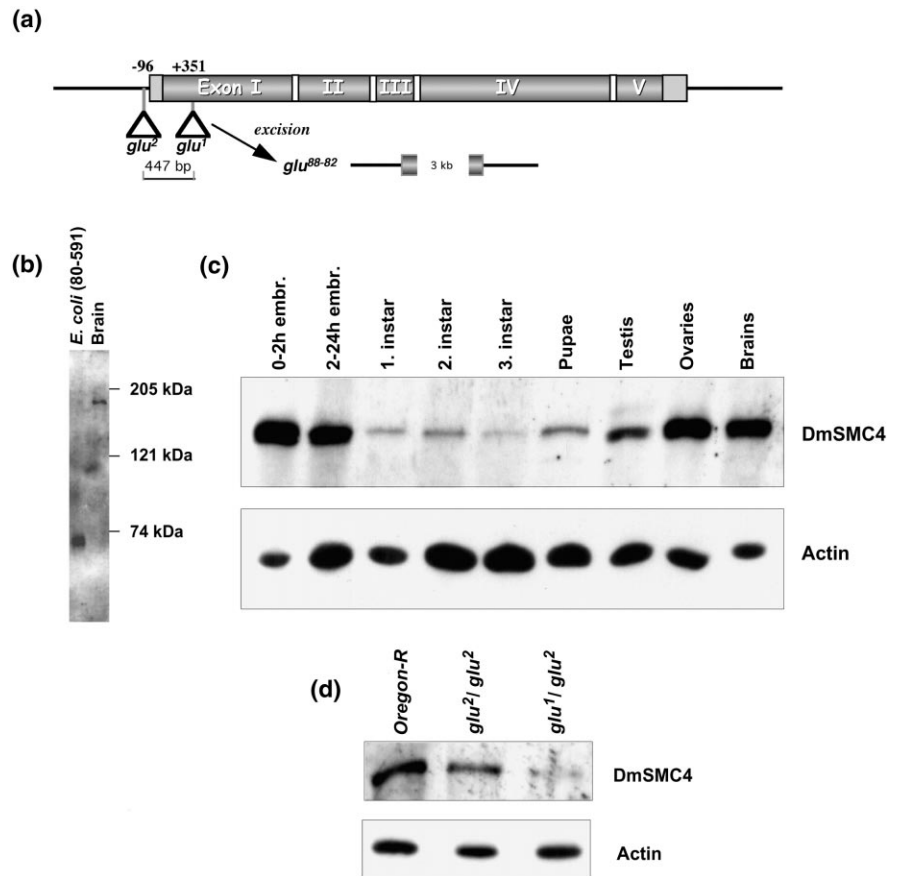
Developmental expression of *DmSMC4* in wild-type and mutant cells

Polyclonal antibodies against an internal fragment of the *DmSMC4* protein were generated and shown to specifically recognize the bacterially expressed polypeptide and a single band of approximately 190 kDa in extracts from third-instar larval brains (Figure 1b). We used the antibodies to determine the levels of expression during different developmental stages and in different tissues (Figure 1c). Although *DmSMC4* was present throughout development, it was most abundant in embryos, third-instar larval brains, and adult ovaries and testes.

Genetic analysis of the mutant alleles showed that *glu¹/glu¹* and *glu⁸⁸⁻⁸²/glu⁸⁸⁻⁸²* were embryonic lethal, *glu¹/glu²* transheterozygotes died as late larvae, and *glu²/glu²* homozygotes died as early pupa. These results suggested a gradual decrease in the level of the *DmSMC4* protein in the different mutant alleles. Probing third-instar larval brain extracts with the affinity-purified *DmSMC4* antibody confirmed these results (Figure 1d). As compared

Figure 1

Genomic organization and Western blot analysis of DmSMC4 expression. **(a)** Genomic map of the *Drosophila* SMC4 locus. The map shows the P element insertion sites in the *glu¹* and *glu²* alleles as well as the lesion in one allele generated by imprecise excision of the *glu¹* P element. **(b)** Characterization of the antibody. A bacterially expressed fragment of DmSMC4 (10 ng; *E. coli* 80–591) and 50 μ g of total third-instar larval brain extract were separated in a 7.5% SDS-PAGE, blotted to nitrocellulose, and probed with crude anti-DmSMC4 serum. **(c)** Developmental Western blots. Approximately 50 μ g of total protein extracts from different developmental stages and tissues were loaded onto 7.5% and 12% SDS gels and probed with anti-DmSMC4 and anti-actin antibodies (as a loading control). **(d)** DmSMC4 expression in mutant third-instar larval brains. Total protein extracts from wild type, *glu²/glu²*, and *glu¹/glu²* were separated as in (c) and probed with immunopurified anti-DmSMC4 antibody.



to the wild-type control, DmSMC4 expression was moderately reduced in *glu²/glu²* and significantly reduced in *glu¹/glu²*. Thus, the P element insertion in *glu¹* probably corresponds to a severe hypomorph allele, while *glu²* is a weak hypomorph. To confirm that the P insertions were responsible for the mitotic phenotypes and lethality, we cloned the complete cDNA into a p-UAS transformation vector [29] and obtained several stable transformants. Driven by a GAL4 insertion (M₂1277), cDNA expression in neuroblasts produced viable [pUAS-Gluon]; *glu²/glu²*; M₂1277 individuals showing no mitotic defects.

Immunolocalization of DmSMC4 and Barren during the cell cycle

We determined the cell cycle distribution of DmSMC4 by simultaneously immunostaining fixed S2 cells with the DmSMC4 antibody (see Materials and methods) and with antibodies against Barren, one of the non-SMC condensin subunits (Figure 2). In interphase, both proteins were present in the cytoplasm (but were not apparently colocalized) and showed low levels of diffuse nuclear staining, excluding the nucleolus (Figure 2a). In prophase, increased nuclear labeling of both proteins showed accumulation with the condensing chromatin (Figure 2b). In pro-

metaphase, both proteins showed discrete colocalization along the condensed chromosome arms, with more intense labeling of the kinetochore regions and the nucleolar organizing region (NOR) of the X chromosome (Figure 2c). To visualize this more clearly, we also show a prometaphase cell stained for DmSMC4 and MPM2 (Figure 2g). In metaphase (data not shown) and early anaphase (Figure 2d) when chromosomes are fully condensed, the labeling was more uniform throughout the core of the chromatids. During later stages of anaphase, both proteins were still associated with the distal ends of segregating chromatids but were excluded from the decondensing centromere-proximal regions (Figure 2e). In telophase, both proteins showed low levels of diffuse nuclear and cytoplasmic staining (Figure 2f). These results show that a major pool of DmSMC4 and Barren undergoes significant relocalization from the cytoplasm to the nucleus during chromosome condensation and back to the cytoplasm as cells exit mitosis. A minor pool appeared to be bound to chromatin independently of cell cycle stage. Similar results were obtained with third-instar larval neuroblasts. To confirm the specificity of the immunofluorescence, we preincubated the antibody with the purified antigen, which abolished all staining patterns (data not shown).

Figure 2

Cell cycle localization of DmSMC4 and Barren in *Drosophila* S2 tissue culture cells. In panels (a–f) all cells are stained for DNA, DmSMC4 and Barren, and in the corresponding merged images DNA is in blue, DmSMC4 is in green, and Barren in red. (a) Interphase cell showing mostly cytoplasmic staining of both DmSMC4 and Barren, although low-level nuclear staining is also observed. The merged image shows that the two antigens do not colocalize in the cytoplasm. (b) Cell in prophase showing increased nuclear staining of both DmSMC4 and Barren. (c) Prometaphase cell showing condensed chromosomes with DmSMC4 and Barren colocalizing to the sister chromatid cores. Note the strong labeling of the NOR of the X chromosome (arrow). (d) Early anaphase cell showing tight colocalization of DmSMC4 and Barren to a coiled axial structure of individual chromatids. (e) Cell in late anaphase showing simultaneous loss of DmSMC4 and Barren from the centromere proximal end of the segregating sister chromatids. (f) Telophase cell showing strong cytoplasmic and low-level nuclear staining of both proteins. We extracted preparations in (a) and (f) with Triton for 10 min after fixation and those in (b–e) during fixation to allow better chromosome staining (see Supplementary materials and methods). (g) Cell in prometaphase stained for DNA (not shown), DmSMC4 (green), and MPM2 (red). The DmSMC4 panel shows staining of the sister chromatid cores and some accumulation of the antigen at the centromeres of several chromosomes. Double immunostaining with MPM2 shows that DmSMC4 at the centromere colocalizes with kinetochores. The scale bar represents 5 μ m.

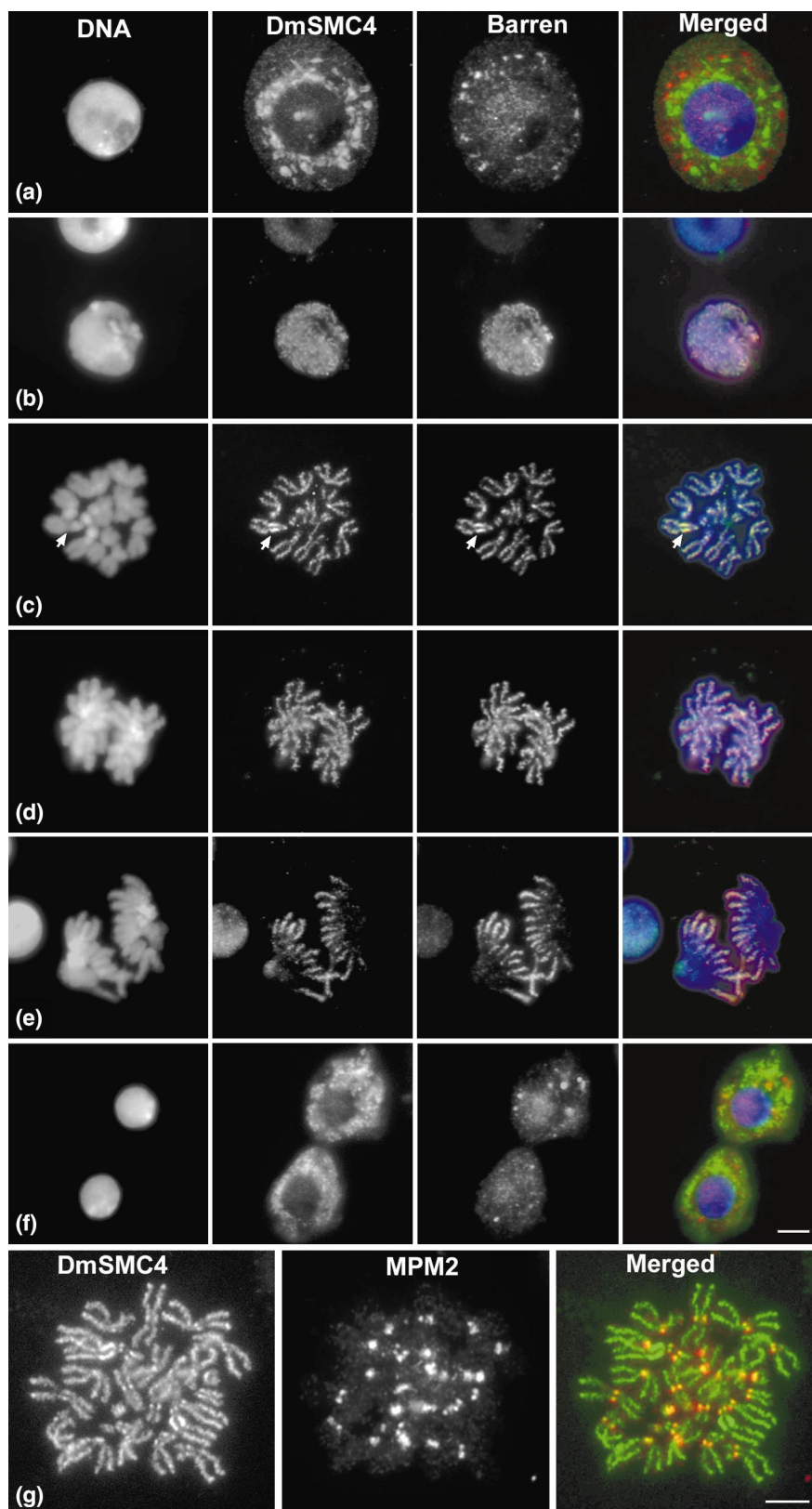
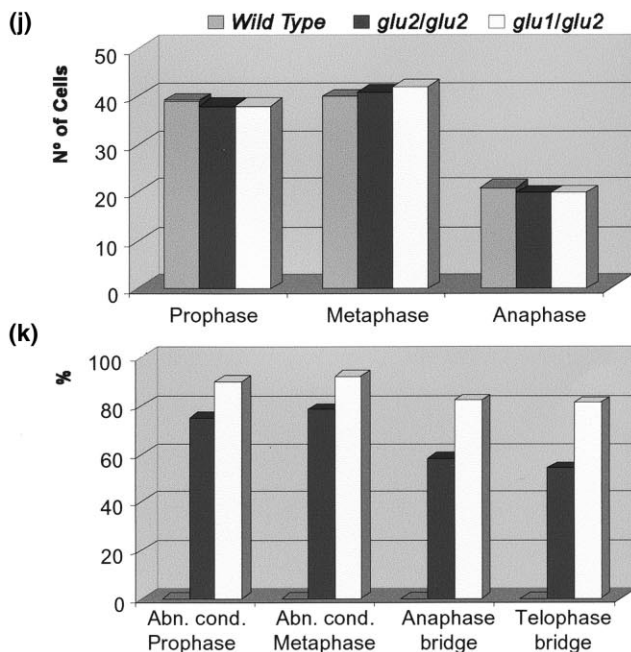
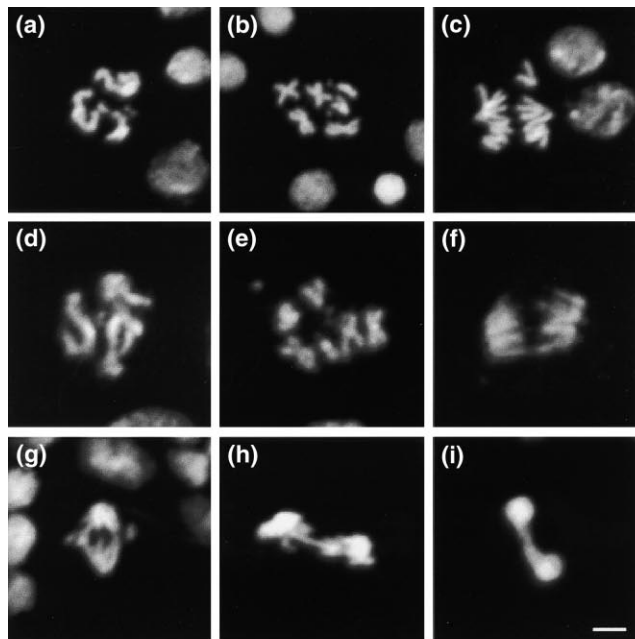


Figure 3

Phenotypic characterization of the *gluon* mutant alleles. For comparison, (a–c) wild-type mitotic figures are shown above representative images from (d–i) *glu2/glu2* cells. (a) Cell in prophase showing paired homologous chromosomes. (b) Prometaphase cell showing tightly condensed chromosomes and well-resolved sister chromatids. (c) Early anaphase cell showing migrating sister chromatids. (d) Mutant cell in prophase showing hypocondensed pairs of homologous chromosomes. (e) Prometaphase cell showing short chromosomes with diffuse chromatin staining and only partial sister chromatid resolution. (f) Early anaphase cell showing diffuse chromatin staining of segregating chromatids. (g,h) Cells in anaphase showing abnormal chromatin condensation, chromatin bridges, and some chromosome fragmentation. (i) Cell in telophase showing extensive chromatin bridges. The scale bar represents 5 μ m.

Mitotic progression of *DmSMC4* mutant cells

We then isolated brains from *glu2/glu2* and *glu1/glu2* third-instar larvae to determine mitotic parameters and phenotype. The mitotic index in wild-type neuroblasts was higher (1.11%) than that in either *glu2/glu2* (0.87%) or *glu1/glu2* (0.83%). Mitotic cells from mutant individuals showed several defects (Figure 3d–i) as compared to wild-type controls (Figure 3a–c). At prophase, when homologous chromosomes in *Drosophila* are known to pair, condensing chromatin appeared fuzzy (Figure 3d). This diffuse appearance was observed from prophase to anaphase and with different DNA dyes (data not shown). In addition, many prometaphase and metaphase figures did not show well-defined sister chromatids (Figure 3e). Most cells in anaphase showed abnormally decondensed chromatin and contained chromatin bridges and some chromosome fragmentation (Figure 3f–h). Chromatin bridges were also observed during telophase (Figure 3i). These chromosome phenotypes are unlikely to be a secondary consequence of dying cells because immunofluorescence analysis with various antibodies against a number of cellular antigens, including checkpoint proteins and mitotic regulators, shows normal distribution (data not shown).

Quantification of mitotic parameters in mutant cells showed that, of the cells in mitosis, the percentage of cells at different stages was similar to that for wild-type controls (Figure 3j). These data indicate that the mutations do not cause a delay in mitotic progression. Most cells in prophase and metaphase had defects in condensation and/or segregation (Figure 3k). These results indicate that the effects of the mutations manifest early in chromosome condensation and that they persist throughout mitosis. Also, a large proportion of the anaphase figures in *glu2/glu2* (58%) and *glu1/glu2* (82%) had chromatin bridges, indicating a direct correlation between condensation defects and the ability to segregate properly in anaphase. Additionally, the presence of chromatin bridges during telophase (54% and 81% respectively) showed that the abnormal chromatin does not resolve during segregation.

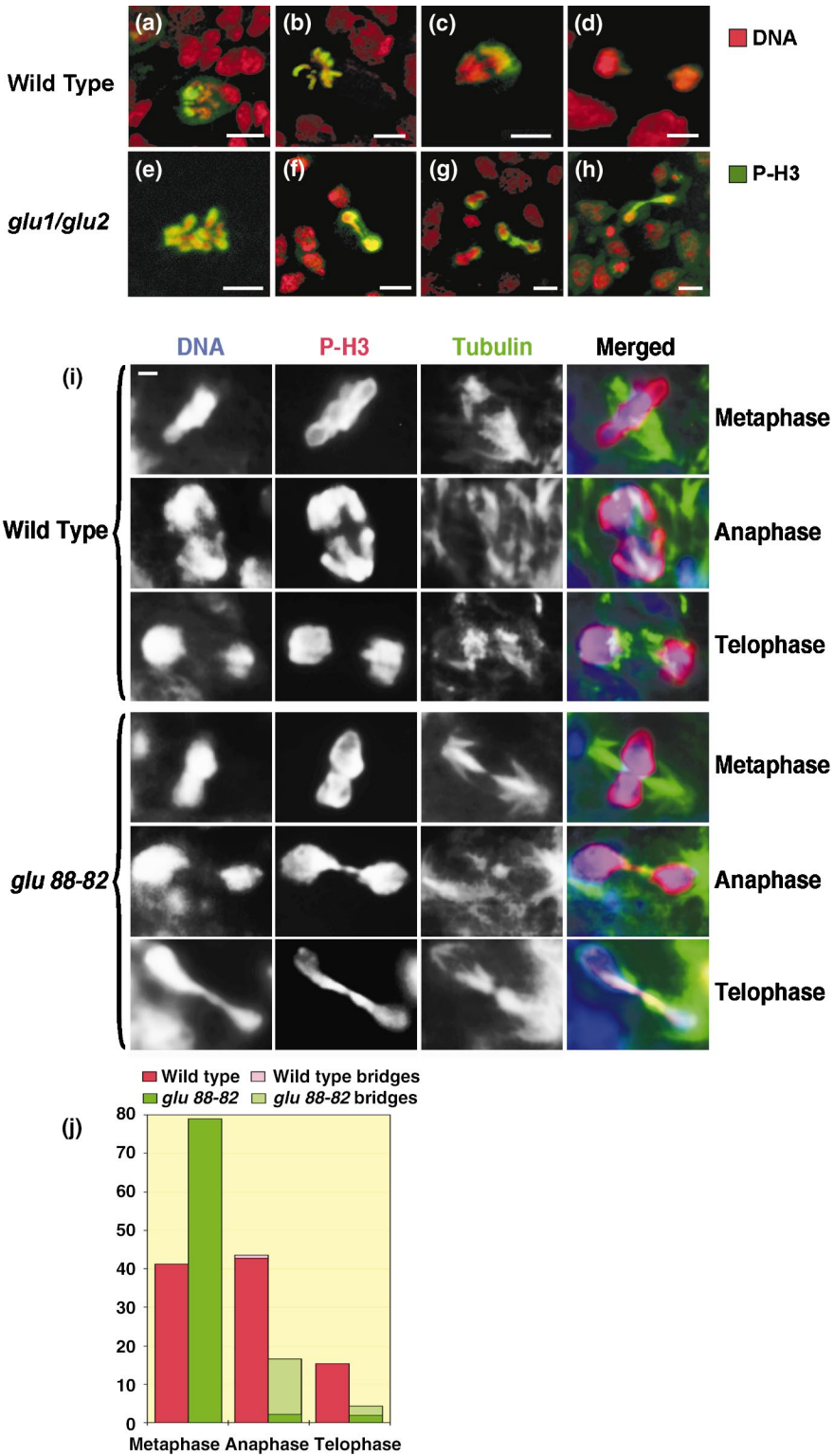
To study abnormal chromosome condensation in anaphases and telophases in more detail, we immunostained wild-type and mutant cells with an anti-phosphohistone H3 antibody (Figure 4a–h). Histone H3 phosphorylation at Ser-10 is highly correlated with the state of chromosome

(j,k) Quantification of mitotic figures in Or-R, *glu2/glu2*, and *glu1/glu2*.

(j) Distribution of mitotic cells in prophase, metaphase, and anaphase as a percentage of the total number of mitotic cells (telophases not included). (k) Quantification of abnormal mitotic figures as a percentage of the total number of figures in each phase. For wild-type, *glu2/glu2*, and *glu1/glu2* cells, 45,700 nuclei (4 brains), 63,000 nuclei (8 brains), and 95,500 nuclei (11 brains) were counted, respectively.

Figure 4

Analysis of chromatin bridges in mutant cells at different developmental stages. We used immunofluorescence to detect the distribution of phosphohistone H3 epitopes (green) during mitosis in (a–h) wild-type and (e–h) *glu¹/glu²* neuroblasts. Note that phosphorylated histone H3 persists in mutant cells up to telophase. The scale bar represents 5 μ m. (i) Images of mitotic chromosomes from the ventral nerve chord of wild-type (top half) and homozygous *gluon⁸⁸⁻⁸²* (bottom half) embryos, stained with DAPI to show DNA and antibodies to α -tubulin and the phosphorylated form of histone H3. Similar anaphase and telophase bridges were also common in embryos in which *gluon⁸⁸⁻⁸²* was combined with deficiencies spanning the SMC4 locus. The scale bar represents 1 μ m. (j) Quantification of mitotic figures. The proportion of wild-type and *glu⁸⁸⁻⁸²* cells in metaphase, anaphase, or telophase as well as the proportion of cells with chromatin bridges are shown. A total of 361 wild-type and 271 *glu⁸⁸⁻⁸²* cells were counted.



condensation [30]. Consistently, anaphase bridges that persisted until telophase were always highly stained, and this result suggests that decondensation had not been completed.

An analysis of mitotic domains was performed in the *glu⁸⁸⁻⁸²/glu⁸⁸⁻⁸²* excision derivative since the phenotype appeared to be more severe than either *glu⁸⁸⁻⁸²/glu¹* or *glu¹/glu¹*. Labeling of wild-type cells for DNA, phosphohistone H3, and tubulin showed the predicted patterns during mitosis (Figure 4i). However, in mutant embryos, anaphases and telophases showed chromatin bridges that were stained by the anti-phosphohistone H3 antibody (Figure 4i). Quantification of the mitotic phenotypes in mutant embryos indicated that a number of cells arrest in metaphase and that the majority of cells reaching anaphase show chromatin bridges (Figure 4j). The fact that embryos show metaphase arrest while larval neuroblasts do not may be because the embryonic alleles are more severe than the larval lethal allele, or it may imply different outcomes when the function of DmSMC4 is compromised. Thus, the mitotic phenotype caused by mutations in *gluon* showed that DmSMC4 was required for proper chromosome segregation in both embryonic and larval tissues.

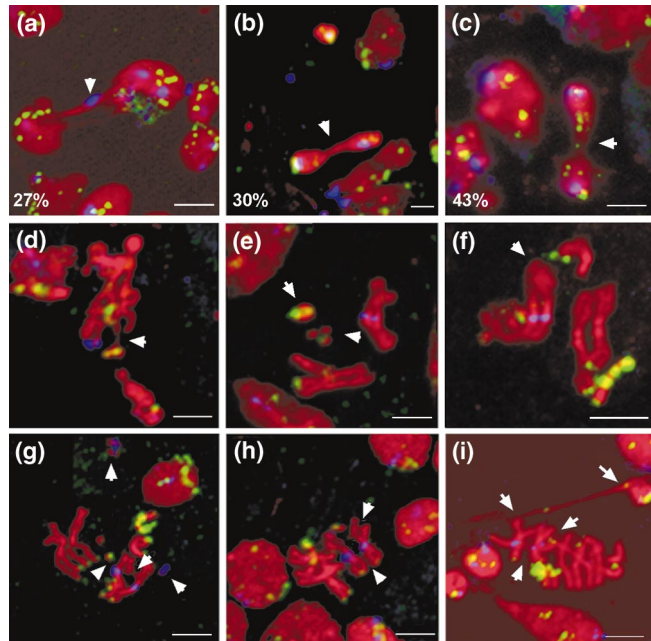
Chromatin bridges, chromosome fragmentation, and apoptosis in *DmSMC4* mutant neuroblasts

Cytological analysis of mutant neuroblasts showed that many mutant cells contained anaphase bridges as well as DNA fragments. In order to determine the type of chromosomal aberrations present in these cells, we performed in situ hybridization with centromeric and telomeric probes (Figure 5). The results indicate that, of the anaphase or telophase cells containing chromatin bridges, only 27% showed centromeric signals in the bridges (Figure 5a), while 43% showed a telomeric signal (Figure 5c) and the remaining 30% contained neither (Figure 5b). These results indicate that in most cases centromeres segregated normally and that chromatin bridges were most frequently associated with telomeres or euchromatin.

We observed prophase and metaphase cells with very irregular chromosome structure and condensation. These included chromosomes with abnormal condensation patterns (Figure 5d,f) and others that did not contain telomeric sequences (Figure 5f,g,h,i). Other alterations/rearrangements included circular Y chromosomes (Figure 5e), pericentromeric/centromeric fragments, and telomeric fragments (Figure 5g). We also observed metaphase chromosomes that appeared to be connected to chromatin masses (Figure 5i). These observations suggest that chromosome fragmentation and chromosome rearrangements may involve different chromosome regions.

Mutant brains were generally smaller than wild-type brains of similar stages. Since the mitotic progression of

Figure 5



Characterization of chromatin bridges and chromosomal rearrangements observed in *DmSMC4* mutant neuroblasts. **(a–c)** In situ hybridization of anaphase and telophase *glu¹/glu²* neuroblasts exhibiting chromatin bridges. Centromeric probes for chromosome 3 (dodeca-satellite) and chromosome 2 (RSP) are shown in blue, and probes for the Het-A I and II telomere transposons are in green. DNA is shown in red. The percentage of anaphases/telophases exhibiting the (a) centromeric, (b) non-centromeric or non-telomeric, and (c) telomeric signals in the chromatin bridges observed is shown. **(d–i)** In situ hybridization showing chromosome rearrangements. *glu¹/glu²* neuroblasts were hybridized with centromeric probes (shown in blue) for (d,h,i) chromosome 2 (RSP) or (e–g) chromosome 3 (dodeca-satellite) and a probe for detecting the telomere transposons Het-A I and II, shown in green in all panels. DNA is shown in red. (d) Cell showing abnormally condensed chromatin and an abnormal chromosome 2 (arrow). (e) Cell showing a circular Y chromosome (arrow at the top) and several chromosome fragments (arrow head at the bottom). (f) Cell showing a third chromosome associated with a large, decondensed chromatin mass (arrow head). (g) Cell showing several small centric chromosome fragments. (h) Cell showing chromosomes that do not show telomere labeling in most chromosomes and abnormal structure of chromosome 2. (i) Cell showing chromosome arms joined to abnormally decondensed chromatin and several linkages between chromosomes. The scale bar represents 5 μm.

mutant neuroblasts was not delayed and the mitotic indices were only moderately lower than those for wild-type controls, this difference could only be explained by the elevated loss of cells during development. Accordingly, *glu²/glu²* and *glu¹/glu²* third-instar larval brains were stained for apoptotic cells. Wild-type brains had a low frequency of apoptotic cells (0.5%), while mutant brains contained a much higher number of apoptotic cells (2.5% in *glu²/glu²* and 3.0% in *glu¹/glu²*). Since chromosome breakage was detected in mutant cells, it appeared most likely that

the increased level of apoptosis was a consequence of irreversible chromosome damage.

Chromosome condensation in *DmSMC4* mutant neuroblasts

The cytological analysis of mutant cells suggested that chromosomes had abnormal condensation patterns (see Figure 3a). Two specific parameters of chromosome morphology were analyzed in detail. We measured the length of chromosome arms to assess whether mutant cells were able to shorten the longitudinal axis of chromosomes, and we also quantified the total number of chromosomes that had clearly defined sister chromatids in order to assess whether proper sister chromatid resolution takes place. We arrested mutant cells in prometaphase with colchicine for different periods of time, and we hybridized them with subtelomeric- and pericentromeric-specific probes to avoid measuring chromosomes that had lost distal fragments (Figure 6a). Only chromosomes that had both pericentromeric and subtelomeric signals were used for the quantification (Figure 6b,c and Table 1). Representative samples of wild-type, *glu²/glu²*, or *glu¹/glu²* cells that were hybridized with probes to chromosome II are shown (Figure 6a). Chromosome length was measured as the number of pixels (0.091 $\mu\text{m}/\text{pixel}$) from the center of the subtelomeric hybridization signal to the center of the pericentromeric signal for each arm, and the two values were added (Figure 6b and Table 1). Overall, the results showed that mean chromosome length (chromosome II and III) for either mutant combination (*glu²/glu²* and *glu¹/glu²*) that had been incubated for different times in colchicine (0, 30, and 60 min) did not differ significantly from that of the wild-type controls. Accordingly, the dynamics of chromosome shortening in these experimental conditions were similar in both wild-type and mutant cells (Table 1).

In order to evaluate if sister chromatid resolution was affected, we counted the number of complete chromosomes that showed clearly resolved sister chromatids (Figure 6c). We found that, in the absence of colchicine incubation, the percentage of mitotic chromosomes in mutant cells showing sister chromatid resolution, *glu²/glu²* (46.8%) and *glu¹/glu²* (32%), was significantly lower than in wild-type controls (75%). As expected from the severity of the alleles, this phenotype was stronger in *glu¹/glu²* cells. Incubation of either wild-type or mutant cells in colchicine for 30 min did not have any effect in terms of resolution. However, incubation for 60 min led to a reduction in the percentage of chromosomes exhibiting visible sister chromatid resolution in both wild-type and mutant cells. This effect was probably a consequence of overshortening the longitudinal axis of chromosomes during mitotic arrest, which thus made it difficult to distinguish sister chromatids. Nevertheless, the differences observed between mutant and wild-type chromosomes could never

be abolished, and this finding suggests that chromatin of mutant cells was not able to compact properly to allow adequate sister chromatid resolution.

Localization of Barren in *DmSMC4* mutant neuroblasts

Since, similar to wild-type cells, chromosomes in mutant neuroblasts retained the ability to shorten their arms with normal kinetics, we wanted to address whether these still contained detectable levels of other condensin subunits that might mediate this process. We chose to use the anti-Barren antibody to determine the distribution of this condensin subunit in mutant cells since it has a much higher titer than do *DmSMC4* antibodies. This higher titer allows visualization of very low protein levels (Figure 7). Wild-type cells show Barren localized throughout the chromosome axis and some accumulation at the centromere (Figure 7a). Mutant cells that show chromosomes with largely resolved sister chromatids contained normal levels of Barren (Figure 7b). However, most mutant cells that displayed abnormal chromatin compaction and partly resolved sister chromatids had reduced amounts of Barren, and this finding suggests that *DmSMC4* may be essential for its proper localization (Figure 7c, top panel). In these cells, the level of sister chromatid resolution was directly related to the intensity of Barren staining. This result suggests an inverse correlation between the level of condensin complex and the severity of the phenotype. Additionally, chromosomes in mutant cells frequently showed most intense labeling of the centromeric region, with labeling intensity decreasing gradually to the telomeres (Figure 7c). Finally, in mutant cells that contained metaphase chromosomes with clearly unresolved sister chromatids, the localization of Barren appeared diffuse (Figure 7c, lower panel). This suggests that the sister chromatid cores did not individualize and that sister chromatid resolution probably requires stoichiometric amounts of the Barren subunit but likely also of the entire condensin complex.

Discussion

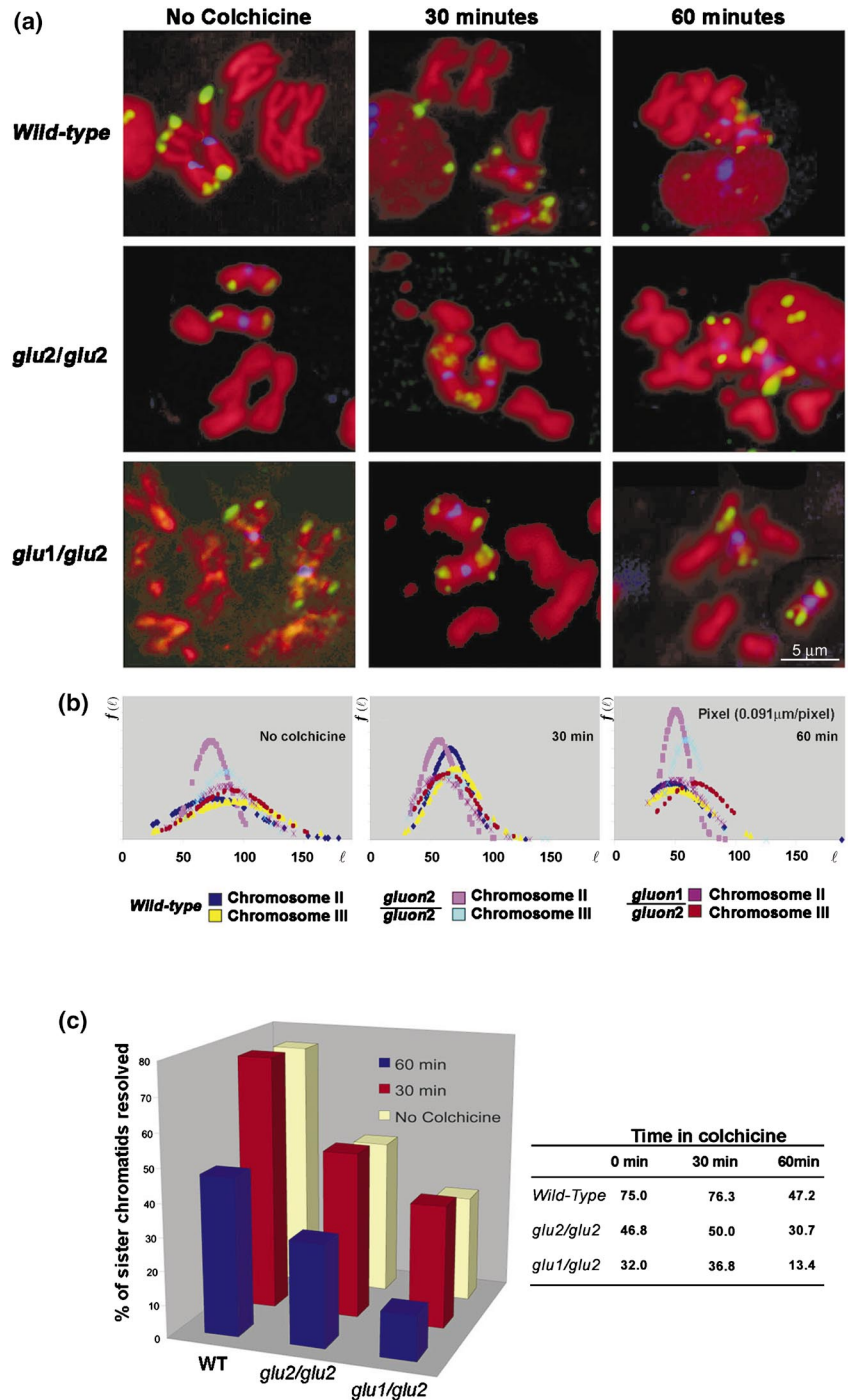
The results presented in this study extend our understanding of the role of the SMC4 protein in mitotic chromosome dynamics. Until now, yeast genetic analyses and *Xenopus* biochemical data have been crucial for the identification and study of the condensin subunits. Here we have been able to exploit the genetics and well-defined cytology of the fruit fly to study the consequences of the loss of the core condensin subunit SMC4 on chromosome structure and mitotic progression in a metazoan organism.

The *gluon* locus encodes the *Drosophila* SMC4 condensin subunit

Our results show that *DmSmc4* encodes a protein that clearly belongs to the SMC4 subfamily of SMC proteins; this protein is one of the two SMC subunits of the condensin complex. In *Xenopus* and yeast, the protein is found

Figure 6

Characterization and quantification of chromosome condensation parameters. **(a)** Representative images of prometaphase chromosomes used for the quantification. Wild-type, *glu²/glu²*, and *glu¹/glu²* neuroblasts, were incubated in colchicine for different times (0, 30, and 60 min) and hybridized with a centromeric probe for chromosome 2 (RSP; shown in blue) and specific sub-telomeric probes for 2L and 2R (shown in green). DNA is stained in red. We also did similar preparations, not shown in this figure, for chromosome 3 by using different probes. **(b)** Frequency distribution [$f(\ell)$] of the length (ℓ) of chromosomes II and III in wild-type, *glu²/glu²*, and *glu¹/glu²* neuroblasts after they were incubated for different times in colchicine. **(c)** Graphical representation of the percentage of chromosomes (shown in Table 1) showing resolved sister chromatids in wild-type, *glu²/glu²*, and *glu¹/glu²* neuroblasts after they were incubated for different times in colchicine. A total of 979 chromosomes from 52 brains were scored. The scale bar represents 5 μm .



in a multi-subunit complex consisting of SMC2/4 as well as CAP-G, -H, and -D2 proteins [16, 20]. DmSMC4 is likely to be found in a similar complex in *Drosophila*, as we have identified all subunit homologs by sequence homology [9], and DmSMC2/DmSMC4/Barren and additional proteins can be coimmunoprecipitated from embryo extracts (Shen and Wu, personal communication).

DmSMC4 is an essential gene, as animals homozygous for either of two P insertion alleles die as embryos (*glu¹*) or as larvae/pupae (*glu²*). The maternal contribution of DmSMC4 is presumably sufficient to sustain embryos through the early syncytial blastoderm cycles. DmSMC4 was found to be expressed in all mitotically active tissues and developmental stages. The highest levels of protein

Table 1

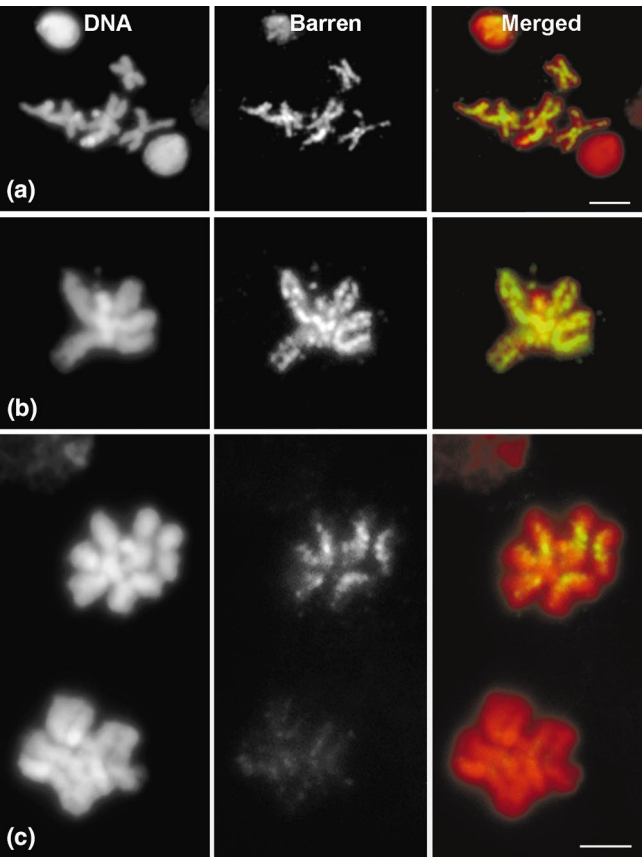
Quantification of chromatid arm length in wild-type and mutant cells after colchicine incubation (μm).

Genotypes	Time in colchicine					
	0 min		30 min		60 min	
	II	III	II	III	II	III
Wild type	6.80 ± 3.23	8.29 ± 3.43	5.94 ± 1.42	6.36 ± 1.81	4.17 ± 2.29	4.52 ± 2.78
<i>Glu¹/glu²</i>	7.60 ± 2.45	8.25 ± 2.72	5.15 ± 2.05	5.81 ± 1.97	4.57 ± 2.16	6.04 ± 2.33
<i>Glu²/glu²</i>	6.59 ± 1.32	7.63 ± 1.91	5.13 ± 1.30	5.72 ± 1.99	4.57 ± 1.02	5.33 ± 1.29

Results for chromosome 2 (II) or chromosome 3 (III) are shown.

were found in tissues containing a high number of proliferating cells, and this observation is consistent with DmSMC4 being required for both mitotic and meiotic cell divisions.

Figure 7



Immunolocalization of Barren in metaphase chromosomes from wild-type and *glu²/glu²* neuroblasts. **(a)** Wild-type neuroblasts in metaphase that show Barren staining throughout the chromosome arms and accumulation at the centromere region. **(b)** Mutant neuroblast showing well-condensed chromosomes with separated sister chromatids and high levels of axial staining. **(c)** Two neighboring cells showing abnormally condensed chromosomes with unresolved sister chromatids and diffuse labeling of the longitudinal axis of the chromosomes. Note the stronger accumulation of Barren at the primary constriction. The scale bar represents 5 μm.

DmSMC4 localizes to mitotic chromosomes

DmSMC4 exhibits a cell cycle distribution expected of a protein involved in the condensation and maintenance of mitotic chromosome structure. It is loaded onto condensing chromosomes early in prophase and dissociates late in anaphase/telophase when the condensation process is reversed. During these stages, the protein shows a restricted distribution running along the longitudinal axis of chromatids, similar to what has been observed for topo II and ScII/SMC2 [12, 31]. A minor pool of DmSMC4 remains associated with chromatin in telophase and interphase, whereas the major fraction becomes cytoplasmic at this time. The initial increase in chromatin association occurred before the onset of nuclear-envelope breakdown, as we observed by costaining for the nuclear lamina (data not shown). While immunostaining for SMC4 has revealed interphase cytoplasmic localization in *S. pombe* [16], in *Xenopus* the protein appears to be nuclear during interphase [13]. The human and chicken CAP-E [SMC2] proteins have also been reported to be nuclear [12, 32]. The differences in interphase localization could be due to differential solubility during fixation procedures of nuclear versus cytoplasmic SMC4. In *Xenopus*, mitosis specific phosphorylation of condensin controls its activity, whereas in *S. pombe* activity appears to be controlled by the shuttling of condensin in and out of the nucleus.

We have additionally shown that Barren colocalizes with DmSMC4 on condensed chromosomes and that the localization of Barren is dependent on the presence of DmSMC4. These findings are consistent with participation in a common complex, as has been shown in *Xenopus* and yeast [13, 16]. This complex may only be formed late in interphase, however, as Barren and DmSMC4 do not appear to colocalize in the cytoplasm. This finding is consistent with the presence of a separate condensin “regulatory” complex containing the non-SMC subunits [33]. On condensed chromosomes, DmSMC4 and Barren are more concentrated in the centromeric region. This observation is consistent with chromosome condensation initiating at the centromere and then spreading distally, similarly to the way in which mitosis-specific phosphorylated forms of histone H3 accumulate [30]. A role for DmSMC4 and Barren in the condensation of the centromeric hetero-

chromatin underlying the kinetochore may be necessary for the proper assembly of the kinetochore. Indeed studies in *S. cerevisiae* have implicated BRN1 in normal kinetochore function [27]. Interestingly, topo II also shows a similar enrichment at centromeres in a number of mammalian cells [34].

DmSMC4 is required for sister chromatid resolution

To analyze in detail the effects of *DmSMC4* mutants on chromosome morphology, we quantified two aspects of chromosome condensation in neuroblasts. Our results unambiguously indicated that the shortening of the longitudinal axis of the chromosome was unaffected in *DmSMC4* mutants and occurred with the same kinetics as in wild-type cells. However, we observed a significant reduction in the ability of *DmSMC4* mutant cells to resolve sister chromatids during prometaphase. Arresting mutant neuroblasts with colchicine to allow more time for the condensation process did not rescue the defect in resolution, while chromosome shortening proceeded as in wild-type cells.

How is the chromosome axis shortened when DmSMC4 levels are reduced? The axial shortening of chromosomes observed in mutant cells suggests that either the residual level of DmSMC4 is sufficient to support this aspect of condensation or other factors are responsible for this process. The fact that the kinetics of length reduction appear to be independent of DmSMC4 levels would argue against DmSMC4 involvement in this process. Several results support the hypothesis that topo II might be the enzyme responsible in part for the shortening of the axes [35]. Firstly, topo II has been shown to be required for the hypercondensation of mitotic chromosomes after the treatment of yeast cells with microtubule-depolymerizing agents [5]. Secondly, blocking mammalian cells with the ICRF topo II inhibitors leads to hypocondensed mitotic chromosomes [36, 37]. Thirdly, topo II is targeted to chromatin independently of the condensin complex in vitro [20]. Alternatively, other members of the condensin complex may still mediate arm shortening.

The fact that *DmSMC4* mutant cells are able to shorten their longitudinal chromosome axes but are unable to properly resolve sister chromatids indicates that these two aspects of chromosome condensation can be separated by mutation. Current models suggest that one purpose of chromosome condensation (probably shortening of the axis) is to displace the topo II catenation/decatenation equilibrium in the direction of decatenation and thereby promote the resolution of DNA catenanes produced during replication [36–38]. In this context, our data suggest that wild-type levels of SMC4 are required to drive topo II toward decatenation and thereby promote sister chromatid resolution. However, the failure of sister chromatid resolution at reduced SMC4 levels could also be due to

the inappropriate maintenance of the cohesion of sister chromatid arms. This interpretation is supported by recent results indicating that cohesion along chromatid arms is removed during early stages of chromosome condensation [39]. The fate of *Drosophila* cohesin components [40] in cells that carry mutations of condensin components will be an important question to address.

Mutations in *DmSMC4* cause abnormal chromosome segregation

DmSMC4 mutations affect not only chromosome condensation but also chromosome segregation. All alleles examined in this study cause chromosome segregation defects, manifest as chromatin bridges in anaphase and telophase. Chromosome segregation defects are likely to be the consequence of aberrant sister chromatid resolution. Studies in yeast of mutations for *top2*, *cut3*, and *cut14* strongly suggest that proper chromosome condensation is essential for normal sister chromatid separation [5, 14]. We believe that the chromatin bridges present in *DmSMC4* mutant cells are unlikely to be resolved, as significant chromosome breakage can be observed. The ultimate consequence of this state is probably the increased level of apoptosis observed in mutant brains.

Materials and methods

Analysis and quantification of mitotic phenotypes

All fly stocks were grown at 25°C in standard media. Oregon-R or Canton-S lines were used as wild-type stocks. To identify homozygous *glu*² or transheterozygous *glu*¹/*glu*² third-instar larvae, we balanced the *glu*¹- and *glu*²-bearing chromosomes over T(2;3)SM5a-TM6B (Cayetano González, personal communication). For the quantification of mitotic figures, we prepared third-instar larval brains by using standard protocols except that we chose timing of the hypotonic treatment (1 min) that would allow us to identify the condensation defects without disrupting anaphase figures. This protocol did not allow distinction between telophase and interphase nuclei in wild-type brains, but in the mutants, telophases were easily recognized due to their elongated morphology. We performed quantification by counting the total number of nuclei in brains. To identify embryos homozygous for *glu*⁸⁸⁻⁸², we balanced the mutant chromosomes over CyO, *P*(w⁺mC = *GAL4-Kr.C*)*DC3*, *P*(w⁺mC = *UAS-GFP.S65T*)*DC7* [41]. One hour collections of embryos were aged for 12 hr, dechorionated, and then sorted under a fluorescence dissecting microscope. The sorted embryos were fixed in 40% EM grade paraformaldehyde for 3 min and devitellinized in methanol prior to staining for α -tubulin, phosphohistone H3, and DNA.

Generation of antigen and antibody

We produced a polyhistidine (6xHis)-tagged fragment of DmSMC4 corresponding to residues 80–303 by cloning a NcoI/BglII fragment of the cDNA into the pET23d expression vector (Novagen) and expressing it in *E. coli* BL21(DE3). The protein was partially purified on Ni-agarose columns (Qiagen) before it was applied to SDS-PAGE. Purified protein was obtained from gel slices and injected into rabbits and sheep (Scottish Antibody Production Unit). The anti-DmSMC4 antibodies were used for Western blotting and immunofluorescence as whole serum (1:1000 and 1:500 respectively) or were affinity purified on antigen-coated nitrocellulose strips (1:100 and 1:5 respectively), with similar outcomes.

Quantification of chromosome condensation

For quantitative studies of arm length and sister chromatid separation, brains were incubated in 10 μ M colchicine for 0, 30, and 60 min. They were then treated with a hypotonic solution for 3 min before fixation with

acetic acid, as previously described [42]. Constant-size (59 × 40 mm) images were collected with a Confocal Microscope MRC600. Quantification analysis was performed with the University of Texas Health Science Centre Image Tool version 2.00 for Windows (<http://ddstdx.uthscsa.edu/dig/download.html>).

Supplementary material

Supplementary material including additional materials and methods is available with the electronic version of this article at <http://current-biology.com/supmatin.htm>.

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References

1. Heck MMS: **Condensins, cohesins, and chromosome architecture: how to make and break a mitotic chromosome.** *Cell* 1997, **91**:5-8.
2. Koshland D, Strunnikov A: **Mitotic chromosome condensation.** *Ann Rev Cell Dev Biol* 1996, **12**:305-333.
3. Earnshaw WC, Halligan B, Cooke CA, Heck MMS, Liu LF: **Topoisomerase II is a structural component of mitotic chromosome scaffolds.** *J Cell Biol* 1985, **100**:1706-1715.
4. Gasser SM, Laroche T, Falquet J, Boy de la Tour E, Laemmli UK: **Metaphase chromosome structure: involvement of topoisomerase II.** *J Mol Biol* 1986, **188**:613-629.
5. Uemura T, Ohkura H, Adachi Y, Morino K, Shiozaki K, Yanagida M: **DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*.** *Cell* 1987, **50**:917-925.
6. Adachi Y, Luke M, Laemmli UK: **Chromosome assembly in vitro: topoisomerase II is required for condensation.** *Cell* 1991, **64**:137-148.
7. Hirano T, Mitchison TJ: **Cell cycle control of higher-order chromatin assembly around naked DNA in vitro.** *J Cell Biol* 1991, **115**:1479-1489.
8. Hirano T, Mitchison TJ: **Topoisomerase II does not play a scaffolding role in the organization of mitotic chromosomes assembled in *Xenopus* egg extracts.** *J Cell Biol* 1993, **120**:601-612.
9. Cobbe N, Heck MMS: **SMCs in the world of chromosome biology: from prokaryotes to eukaryotes.** *J Struct Biol* 2000, **129**:123-143.
10. Hirano T: **SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates?** *Genes Dev* 1999, **13**:11-19.
11. Strunnikov AV, Larionov VL, Koshland D: **SMC1: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family.** *J Cell Biol* 1993, **123**:1635-1648.
12. Saitoh N, Goldberg IG, Wood ER, Earnshaw WC: **ScII: an abundant chromosome scaffold protein is a member of a family of putative ATPases with an unusual predicted tertiary structure.** *J Cell Biol* 1994, **127**:303-318.
13. Hirano T, Mitchison TJ: **A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro.** *Cell* 1994, **79**:449-458.
14. Saka Y, Sutani T, Yamashita Y, Saithoh S, Takeuchi M, Nakaseko Y, et al.: **Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis.** *EMBO J* 1994, **13**:4938-4952.
15. Strunnikov AV, Hogan E, Koshland D: **SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family.** *Genes Dev* 1995, **9**:587-599.
16. Sutani T, Yuasa T, Tomonaga T, Dohmae N, Takio K, Yanagida M: **Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4.** *Genes Dev* 1999, **13**:2271-2283.
17. Guacci V, Koshland D, Strunnikov A: **A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*.** *Cell* 1997, **91**:47-57.
18. Losada A, Hirano M, Hirano T: **Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion.** *Genes Dev* 1998, **12**:1986-1997.
19. Michaelis C, Ciosk R, Nasmyth K: **Cohesins: chromosomal proteins that prevent premature separation of sister chromatids.** *Cell* 1997, **91**:35-45.
20. Hirano T, Kobayashi R, Hirano M: **Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a *Xenopus* homolog of the *Drosophila* barren protein.** *Cell* 1997, **89**:511-521.
21. Kimura K, Hirano T: **ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation.** *Cell* 1997, **90**:625-634.
22. Kimura K, Rybenkov VV, Crisona NJ, Hirano T, Cozzarelli NR: **13S condensin actively reconfigures DNA by introducing global positive writhe: implications for chromosome condensation.** *Cell* 1999, **98**:239-248.
23. Kimura K, Hirano M, Kobayashi R, Hirano T: **Phosphorylation and activation of 13S condensin by Cdc2 in vitro.** *Science* 1998, **282**:487-490.
24. Bhat MA, Philp AV, Glover DM, Bellen HJ: **Chromatid segregation at anaphase requires the *barren* product, a novel chromosome-associated protein that interacts with topoisomerase II.** *Cell* 1996, **87**:1103-1114.
25. Holm C, Goto T, Wang JC, Botstein D: **DNA topoisomerase II is required at the time of mitosis in yeast.** *Cell* 1985, **41**:553-563.
26. Lavoie BD, Tuffo KM, Oh S, Koshland D, Holm C: **Mitotic chromosome condensation requires Brn1p, the yeast homologue of Barren.** *Mol Biol Cell* 2000, **11**:1293-1304.
27. Ouspenski II, Cabello OA, Brinkley BR: **Chromosome condensation factor Brn1p is required for chromatid separation in mitosis.** *Mol Biol Cell* 2000, **11**:1305-1313.
28. Kania A, Salzberg A, Bhat M, D'Evelyn D, He Y, Kiss I, et al.: **P-element mutations affecting embryonic peripheral nervous system development in *Drosophila melanogaster*.** *Genetics* 1995, **139**:1663-1678.
29. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118**:401-415.
30. Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, et al.: **Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation.** *Chromosoma* 1997, **106**:348-360.
31. Earnshaw WC, Heck MMS: **Localization of topoisomerase II in mitotic chromosomes.** *J Cell Biol* 1985, **100**:1716-1725.
32. Schmiesing JA, Ball ARJ, Gregson HC, Alderton JM, Zhou S, Yokomori K: **Identification of two distinct human SMC protein complexes involved in mitotic chromosome dynamics.** *Proc Natl Acad Sci USA* 1998, **95**:12906-12911.
33. Kimura K, Hirano T: **Dual roles of the 11S regulatory subcomplex in condensin functions.** *Proc Natl Acad Sci USA* 2000, **97**:11972-11977.
34. Rattner JB, Hendzel MJ, Furbee CS, Muller MT, Bazett-Jones DP: **Topoisomerase II α is associated with the mammalian centromere in a cell cycle- and species-specific manner and is required for proper centromere/kinetochore structure.** *J Cell Biol* 1996, **134**:1097-1107.
35. Warburton PE, Earnshaw WC: **Untangling the role of DNA topoisomerase II in mitotic chromosome structure and function.** *Bioessays* 1997, **19**:97-99.
36. Gimenez-Abian JF, Clarke DJ, Mullinger AM, Downes CS, Johnson RT: **A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes.** *J Cell Biol* 1995, **131**:7-17.
37. Gorbisky GJ: **Cell cycle progression and chromosome segregation in mammalian cells cultured in the presence of the Topoisomerase II inhibitors ICRF-187 [(+)-1,2-bis(3,5-dioxypiperazinyl-1-yl)propane; ADR-529] and ICRF-159 (Razoxane).** *Cancer Res* 1994, **54**:1042-1048.
38. Holm C: **Coming undone: how to untangle a chromosome.** *Cell* 1994, **77**:955-957.

39. Waizenegger IC, Hauf S, Meinke A, Peters JM: **Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase.** *Cell* 2000, **103**:399-410.
40. Warren WD, Steffensen S, Lin E, Coelho P, Loupart M-L, Cobbe N, *et al.*: **The *Drosophila* RAD21 cohesin persists at the centromere region in mitosis.** *Curr Biol* 2000, **10**:1463-1466.
41. Casso D, Ramirez-Weber F, Kornberg TB: **GFP-tagged balancer chromosomes for *Drosophila melanogaster*.** *Mech Dev* 2000, **88**:229-232.
42. Carmena M, Abad JP, Villasante A, Gonzalez C: **The *Drosophila melanogaster* dodecasatellite sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis.** *J Cell Sci* 1993, **105**:41-50.